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Research Article

Exploring the Potential of *Dyadobacter fermentans* JDP9 Biosurfactants an Antibacterial and Antifouling Biocompatible Agent

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ABSTRACT

This study investigated the antibacterial and antiadhesive properties of a biosurfactant (BS) produced by *Dyadobacter fermentans* JDP9 against various gram-positive and gram-negative bacteria. The presence of BS was confirmed using an oil spreading test, drop collapsing test and emulsification test. The BS demonstrated antimicrobial effects against *Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis* at a 500 mg/mL concentration. Using a 96-well microtiter plate, the results indicated that both antibacterial and anti-adhesive activities increased with higher BS concentrations. Glass slides coated with BS showed significantly reduced biofilm formation compared to control slides. The BS inhibited 71% of *P. aeruginosa* biofilm formation on a medical-grade catheter. Vero cells treated with BS exhibited moderate morphological changes with an IC_{50} value of 217.5 µg/mL, indicating biocompatibility at 200 µg/mL. These findings suggest that BS, with its antibacterial, anti-adhesive, and biocompatible properties, holds potential as a biodegradable antifouling agent.

Keywords: Antifouling agent, Antibacterial agent, Biosurfactant, Biomedical application, glycoprotein.

INTRODUCTION

Biofilms are complex multicellular microbial communities that adhere to biotic or abiotic surfaces and are encapsulated within a self-produced polymeric matrix. The matrix comprises proteins, lipids, polysaccharides, water, extracellular DNA, and various soluble compounds.[1] Both advantageous and detrimental consequences mark the existence of biofilms.[2]

Biofilms are the primary cause of biofouling – unwanted accumulation and growth on a surface–posing significant challenges in medical and industrial settings due to their resilience and capacity to withstand conventional antimicrobial interventions. This issue is crucial in marine transport, water purification, the food industry, healthcare, and biomedical device manufacturing.[2-5] In healthcare, biofilm-related infections contribute to various conditions such as tooth decay, endocarditis, cystic fibrosis, chronic non-healing wounds, meningitis, kidney infections, and complications with implants and prosthetics.[3] The contamination of advanced medical devices and voice prostheses, including *Staphylococcus aureus, Streptococcus viridans, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Proteus mirabilis*, *Pseudomonas aeruginosa* are major concern.[6-9] Current antimicrobial agents often encounter challenges in effectively disrupting the biofilm architecture, thus necessitating the exploration of alternative approaches.[10]

Conventional drugs used in biomedical applications often cause severe cytotoxic effects and struggle against multidrug-resistant pathogens.[11] Furthermore, many antifouling agents resist degradation due to their complex structure, which can take centuries to degrade due to their complex structures.[12] Additionally, current antimicrobials frequently fail to disrupt the biofilm matrix, underlining the need for alternative approaches.[10] Consequently, developing economically and socially acceptable green alternatives is imperative to ensure human health and environmental sustainability.

Biosurfactants (BS) are naturally occurring amphiphilic molecules that microorganisms like bacteria, yeast, and fungi produce. BSs are emerging as promising anti-biofouling agent alternatives due to their ability to prevent biofilm formation and inhibit pathogen attachment on surfaces.[13] BS hinder microbial attachment and disrupt the processes of microbial adhesion and desorption by increasing the hydrophobic attributes of surfaces.[14,15] Their environmental stability, biodegradability, cytocompatibility, non-toxicity, and stability under variable pH and temperature conditions position them as an eco-friendly 'green toolbox' for diverse industrial applications, including food, pharmaceuticals, medicines, dairy, bioprocessing, petroleum, and agriculture.[16-20] Additionally, BS demonstrates potential inhibitory activity against various gram-positive and gram-negative bacteria, fungi, viruses, malarial parasites, and cancer cell lines.[21-24] Notable examples include surfactin and iturin produced by *B. subtilis,*[25] mannosyl erythritol lipids from *Candida antarctica,*[26] and rhamnolipids from *P. aeruginosa,*[27] all of which highlight the broad potential of BS in the medical and industrial setting.

This study provides a detailed analysis of the antibiofilm and antiadhesive properties of BS produced by *Dyadobacter fermentans* JDP9. It evaluates the effectiveness of BS in disrupting and preventing biofilm formation on medical-grade implants, which could have substantial implications for improving healthcare outcomes associated with implant use.

MATERIAL AND METHODS

Culture Maintenance Dyadobacter fermentans JDP9 (NCBI accession number PRJNA735512)

The bacterial culture JDP9 used in the present study was isolated from crude oil-contaminated automobile workshop soil (Anand, Gujarat, India, N 22o 563855' and E 72o 899390'). It was morphologically characterized using gram staining and biochemically characterized using a commercially available biochemical identification kit (HiMedia, Mumbai). Results were recorded after 24 to 48 hours by adding the indicator reagents in a specific test according to the kit manual.

Test Organisms

The bacterial strains utilized in this study include *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Streptococcus faecalis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. All bacterial cultures were stored at -20°C in a suitable medium supplemented with 20% v/v glycerol. Before use, the bacterial cultures were grown overnight in appropriate broth at 37°C under aerobic conditions.

Production and Purification of the BSfrom JDP9 isolate

Inoculum was prepared by inoculating a well-grown isolated colony of JDP9 in trypticase soya broth (TSB) incubated at 30℃. Later, 1% (v/v) of the inoculum was added in previously optimized Bushnell Hass medium (BHM) supplemented with 3.2 gm% potato-peel powder and 4 gm% urea (pH 7.0) for production [28]. The production process was carried out at 30°C for 4 days, maintained under 150 rpm shaking conditions. Cells were removed by centrifuging at 5000 × g for 20 minutes, and the pH of the cell-free broth was adjusted to 2.0 using 6 N hydrochloric acid (HCl), followed by incubation at 4°C till visible precipitates were observed. Precipitates were collected by centrifugation at 5000×g for 10 minutes at 4°C in a cooling centrifuge (Eppendorf Centrifuge 5430 R) and were purified by gel permeation chromatography. Sephadex G–50 was as a stationary phase maintained in a 300 × 10 mm glass column and phosphate buffer (pH 7.0) as the mobile phase. The fractions of the mobile phase were collected and eluted at a 1-mL/min flow rate.

Preliminary Confirmatory Test for BS

An oil spreading test, emulsification test, and surface tension reduction test were performed according to the method suggested by Patel and Nataraj 2016[29] to confirm the BS presence in precipitates.

Antimicrobial Activity of the BS

The antimicrobial activity of BS was evaluated qualitatively by the agar-well diffusion method and quantitatively by microdilution assay in a 96-well plate, as follows.

Agar well Diffusion Method

Each test bacterial culture was spread on sterile Muller-Hinton agar plates using a sterile cotton swab aseptically. The wells (5 mm in diameter) were punctured in the agar plate using a sterile stainless-steel cup-borer and filled with 20 µL of the BS (500 mg/mL). All the plates were incubated at 37℃ for 24 hours. The zone of inhibition surrounding the wells was calculated.[30]

Microdilution Assay in a 96-well Plate

The antimicrobial activity of the BS by micro-dilution method was performed in 96-well flat-bottom tissue culture plates according to the method suggested by Gudina *et al*. 2010[31] and Vecino *et al*. 2018.[32] Briefly, 125 µL of sterile double-strength TSB growth medium was added in the well-1 of the microplates. To this, 125 µL of BSat the concentration of 100 mg/mL was added and diluted until the ninth well (where the BS was 0.39 mg/mL). A 2.5 µL of the overnight grown test microorganisms (OD_{600}= 0.6) were added to all the wells. Well-11 was filled with TSB medium, which served as a negative control, and well-12 was filled with 125 µL TBS medium and 2.5 µL bacterial inoculum without BS, which served as a positive control. The microplates were covered and incubated for 48 hours at 37℃, and the optical density of each well was measured at 600 nm in a microtiter plate reader. The growth inhibition percentages at different BS concentrations for each strain were calculated using the following equation:

$$\text{Growth inhibition c (\%)} = \left(1 - \left(\frac{ODc}{ODo}\right)\right) \times 100$$

OD_c represents the well's optical density with a BS concentration 'c,' and OD_0 is the optical density of the control well (without BS). The assay was performed in triplicates for all concentrations of the BS.

Anti-adhesive Activity of the BS using the Microtiter Plate

To evaluate the anti-adhesive properties of BS, a sterile 96-well flat-bottom microtiter plate was utilized, and 200 µL of the crude solution was serially diluted from 100 to 0.39 mg/mL in PBS into a series of wells, as mentioned previously. The plate was then incubated at 4℃ for 18 hours and subjected to three rounds of washing with PBS. Control wells contained PBS exclusively. Each well was loaded with 200 µL of the test microorganism ($OD6_{00}$ = 0.6) suspended in PBS, and this mixture was incubated at 4℃ for 24 hours. Unattached microorganisms were removed by performing three PBS washes and fixation using 200 µL of 99% methanol for 15 minutes. Following this, the plates were stained with 200 µL of 1% crystal violet for 5 minutes, and excess stain was removed by rinsing the plates with running tap water. Subsequently, the plates were allowed to air-dry. The dye attached to the adherent microorganisms was dissolved in 200 µL of 33% glacial acetic acid, and the optical density was measured at 595 nm. The inhibition percentages for each microorganism at various concentrations of BS were determined using the following formula:

$$\text{Microbial inhibition (\%)} = \left(1 - \left(\frac{ODc}{ODo}\right)\right) \times 100$$

Where OD_c represents the optical density of the well with a BS concentration 'c' and OD_0 is the optical density of the control well (without BS). Triplicate assays were performed at all BS concentrations for each strain.

Anti-biofilm Activity of the BSon the Glass Slide

The BS's effectiveness in preventing biofilm formation was investigated on glass surfaces. Biofilms were cultivated on pre-sterilized microscopic glass slides. In this process, 200 µL of test organisms grown overnight were introduced into sterile petri dishes containing 20 mL of Luria-Bertani (LB) broth. The pre-sterilized microscopic glass slides were submerged in this medium and incubated at 37℃ for 24 hours. Subsequently, the glass slides were removed and transferred to a sterile LB medium containing BS at a 500 mg/mL concentration. They were then incubated for an additional 30 minutes at 28°C. For comparison, biofilms were also developed on pre-sterilized microscopic glass slides not treated with BS, serving as a control. To assess biofilm disruption, all the glass slides were stained with 1% crystal violet and washed with distilled water thrice. The disruption of the biofilms was observed by examining the slides under a microscope.

Anti-biofilm Activity of the BSon Medical grade catheter

Antibiofilm activity by the BS in medical grade catheters (Poly Medicure suction catheter, India)was determined and visualized according to the method described by Salman and Khudair 2015[33] and Satpute *et al*. 2018.[34] The test catheter was first coated with BS (500 mg/mL), the positive test catheter was inoculated with SDS (500 mg/mL), and the third catheter was untreated, which served as a negative control. All three catheters were left at 4℃ overnight. About 5 mL of the overnight-grown bacterial culture of *P. aeruginosa* was inoculated into all three catheters. The catheter tubes were capped at both ends and incubated at 37℃ for 48 hours. After incubation, the culture was removed from all the catheters, rinsed with distilled water, and dried at room temperature for 15 to 20 minutes. After drying, 1-mL of crystal violet (1% w/v) was added to all the catheter tubes and left at room temperature for 20 minutes. All biofilms developed on the catheter surface appeared purple due to staining. Further, the excess stain was removed by washing with 95% ethanol 3 times; the wash solution was collected and read spectrophotometrically at 595 nm. The percentage of biofilm inhibition was calculated using the equation described below. The catheter was further washed with distilled water, allowed to air dry for 30 minutes, and then examined for the intensity of the biofilm.

$$\text{Microbial inhibition c } (\%) = \left(1 - \left(\frac{ODc}{ODo}\right)\right) \times 100$$

Where ODc represents the optical density of the well with a BS concentration 'c' and OD_0 is the optical density of the control well (without BS).

In-vitro cell cytotoxicity assay of the BS

The BS isolated from JDP9 was tested for *in-vitro* cytotoxicity using Vero cells by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide(MTT) assay. Briefly, the cultured Vero cells were harvested by trypsinization and pooled in a 15 mL tube. Then, the cells were plated at a density of 1×10^5 cells/mL (200 µL) into the 96-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24 to 48 hours at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the BS sample in a serum-free DMEM medium. Each sample was replicated three times, and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hours. After the incubation period, MTT (20 µL of 5 mg/mL) was added to each well, and the cells were incubated for another 2 to 4 hours until purple precipitates were visible under an inverted microscope. Finally, the medium and MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µL). Furthermore, DMSO (100 µL) was added to dissolve formazan crystals, and the plate was shaken for 5 minutes. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA), and the percentage cell viability and IC_{50} value were calculated using GraphPad Prism 6.0 software (USA).

Statistical Analysis

All statistical analyses concerning the antimicrobial activity were conducted through the utilization of IBM SPSS (v. 19.0). The data gathered underwent examination *via* one-way analysis of variance (ANOVA), with significant variances being assessed employing Turkey's multiple comparison tests at a confidence level of 95%. Post-hoc analyses were employed to distinguish variations among the groups.

RESULTS AND DISCUSSION

Production and Preliminary Confirmatory Test for BS

Under (previously) optimized conditions and media, BS production yielded 5.98 gm%. The purified product exhibited positive responses in the drop collapsing assay, oil spreading assay (27.22 ± 0.17 mm^2), and emulsification index ($43.62 \pm 2.55\%$) (Fig. 1).

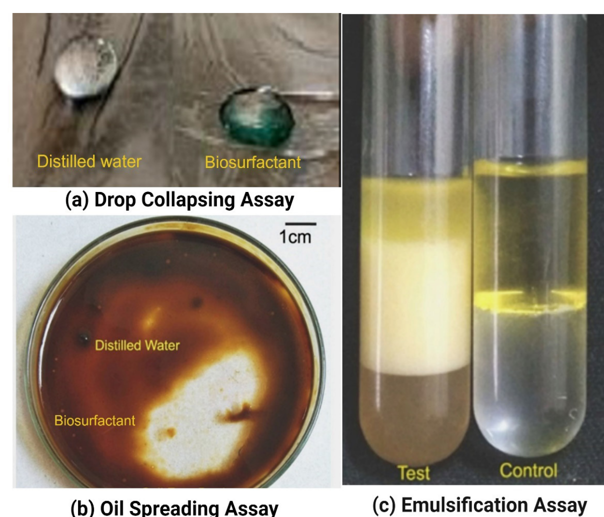

Fig. 1: Preliminary confirmatory tests of BS: **(a)** Drop collapsing assay, **(b)** Oil spreading assay, and **(c)** Emulsification assay.

Antimicrobial Activity on Muller Hinton Agar Plate

The antimicrobial activity of the BS produced by JDP9 at a concentration of 500 mg/mL was tested against various Gram positive and Gram negative bacteria. The tested organisms were *S. aureus, B. subtilis, S. faecalis, E. coli, E. aerogenes, P. aeruginosa, Proteus vulgaris, Serratia marcescens,* and *K. pneumoniae* species. Among the tested bacteria, *E. aerogenes, K. pneumoniae, B. subtilis, S. marcescens, S. faecalis,* and *S. aureus* were found to be more sensitive, as indicated by the area of zone of inhibition with 18.6 ± 0.18, 16.6 ± 0.05, 14 ± 0.08, 12.3 ± 0.05, 10.2 ± 0.08, and 10 ± 0.08 mm^2, respectively (Fig. 2).

Antimicrobial and Anti-adhesive activity of BS in 96-well microtiter well plate

To evaluate the ability of BS to inhibit *in-vitro* biofilm formation, concentrations ranging from 0.39 to 100 mg/mL were tested using 96-well plates.[35] The highest inhibition exhibited by (100 mg/mL) BS was against *S. faecalis* (97.00 ± 2.61mm^2), followed by *P. aeruginosa* (96.46 ± 2.65 mm^2), *E. aerogenes* (95.83 ± 4.55 mm^2), *S. marcescens* (88.93 ± 4.84 mm^2), *K. pneumonia* (87.30 ± 1.28 mm^2), *B. subtilis* (87.19 ± 3.59 mm^2), *S. aureus* (85.43 ± 5.81 mm^2), *E. coli* (85.02 ± 2.15 mm^2), and *P. vulgaris* (80.82 ± 1.56 mm^2) (Table 1).

The BS exhibited the most significant antiadhesive activity against *E. coli* and *S. faecalis* (~97% inhibition), followed by *P. aeruginosa* (~96% inhibition), *P. vulgaris* (~92% inhibition), *S. marcescens* (~89% inhibition), and *S. aureus, E. aerogenes,* and *K. pneumonia* (all showed ~88% inhibition). The lowest inhibition was observed against *B. subtilis* (~50%) at 100 mg/mL (Table 2).

Anti-biofilm activity of BS on the glass slide

Two sterile glass slides were used to test the BS's anti-biofilm effect. One set of slides was used as a control (coded as 'C') without any BS coating, while the other set was coated with a concentration of 500 mg/mL of BS. The control set showed a massive biofilm on the glass slide surface, whereas the BS-coated set (coded as 'E') showed 71% inhibition in biofilm formation. The microscopic observation of slides indicated that the biofilms formed on the glass surfaces were efficiently disrupted by the BS (Fig. 3).

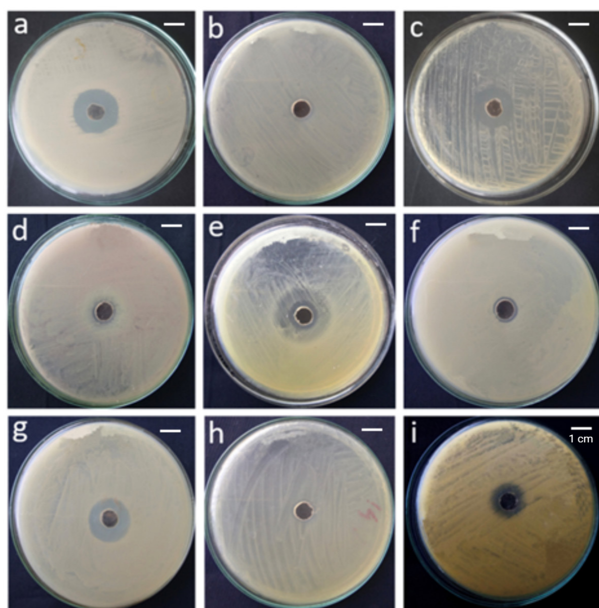

Fig. 2: Antimicrobial effect of BS produced from *Dyadobacter fermentans* JDP9 against **(a)** *B. subtilis,* **(b)** *E. coli,* **(c)** *S. aureus,* **(d)** *S. marcescens,* **(e)** *E. aerogenes,* **(f)** *K. pneumoniae,* **(g)** *S. faecalis,* **(h)** *P. vulgaris*, and **(i)** *P. aeruginosa.*

Anti-biofilm activity on medical grade catheter

In this study, we have used commercially available medical-grade catheters to explore the effect of biosurfactants on bacterial biofilms. As seen in Fig. 4, the untreated catheters were purple due to crystal violet staining in tests. In contrast, the treated catheters showed no purple color, indicating that the BS had inhibited biofilm formation by *P. aeruginosa*.

In-vitro cell cytotoxicity assay of the BS

In-vitro cytotoxicity of BS produced by JDP9 was evaluated on the Vero cell line through an MTT assay. The morphological features of BS-treated and SDS-treated Vero cells at different concentrations (10, 80, 100, and 500 µg/mL) are shown in Fig. 5. Verocells adhered to the plates and retained their original morphology. After the treatment, cells showed a change in morphology. It is clear from the data that as the concentration of BS increased, there was a gradual decrease in cell viability. IC_{50} value of BS was 217.5 µg/mL, indicating that the BS was biocompatible at 200 µg/mL. Results of the present study indicate that BS could be ideal for applying cleaning/coating material for several biomedical equipment.

Strategies aimed at addressing biofouling commonly involve incorporating antimicrobial characteristics into materials to inhibit the attachment of microorganisms and, thereby, the inhibition of biofilm formation. The glycolipid secreted by *Enterobacter cloacae* B14 displayed apparent zones of inhibition ranging from 20.7 to 26.7 mm^2 against Gram positive bacteria and 9.7 to 17.0 mm against Gram negative pathogenic bacteria.[36] Likewise, the BS produced by non-pigmented *Serratia marcescens* NP1 demonstrated inhibition zones measuring 15.0 ± 1.7, 21.0 ± 1.0, and 10.3 ± 0.6 mm^2 against *P. aeruginosa*, *L. monocytogenes* ATCC 13932, and *B. cereus* ATCC 10876, respectively.[37] *Rhodococcus opacus* R7 released (peptide) BS at a concentration of 100 mg/mL, leading to the inhibition of *E. coli* ATCC 29522 and *S. aureus* ATCC 6538 growths with halo zones of 2.6 and 2.7 cm, respectively.[38] Glycolipid BS derived from *Lactiplantibacillus pentosus* demonstrated broad-spectrum antimicrobial activity against food spoilage and topical pathogens.[39] Likewise, BS from *Levilactobacillus brevis* S4 and *Lactoplantibacillus plantarum* S5, isolated from locally fermented milk (pendidam), showed antimicrobial activity against *S. aureus* S1 and *E. coli* E1, helping in stabilizing the cold emulsion of milk chocolate drinks.[40] In the present study, we evaluated the antibacterial activity of BS produced from JDP9 using the crystal violet staining method for qualitative assessment and a 96-well microtiter plate for quantitative analysis. The crystal violet staining method assessed an organism's capacity to form a biofilm on a polystyrene microtiter plate, indirectly measuring the attached bacterial biomass.[41] Utilizing a 96-well microtiter plate provides a cost-effective approach requiring small reagent volumes, enabling multiple tests in a single run.[42] BS demonstrated different levels of antimicrobial and antiadhesive activities against all tested

Table 1: Antimicrobial activity (% growth inhibition) of various concentrations of the BS on the growth of selective microorganisms in 96-well microtiter plate.

BS(mg/mL)	*B. subtilis*	*E. coli*	*S. aureus*	*S. marcescens*	*E. aerogenes*	*K. pneumoniae*	*S. faecalis*	*P. vulgaris*	*P. aeruginosa*
0.39	16.56 ± 1.90 ^{f,C}	11.29 ± 2.10 ^{g,D}	8.94 ± 0.83 ^{f,DE}	21.42 ± 1.48 ^{f,B}	31.25 ± 0.99 ^{f,A}	20.31 ± 1.48 ^{g,B}	31.90 ± 1.92 ^{e,A}	7.53 ± 1.55 ^{f,E}	27.15 ± 1.76 ^{h,B}
0.781	20.49 ± 1.26 ^{f,DE}	23.03 ± 1.44 ^{f,CD}	16.41 ± 0.95 ^{ef,E}	27.35 ± 0.78 ^{ef,C}	56.80 ± 2.05 ^{e,A}	22.47 ± 0.17 ^{g,D}	38.57 ± 0.95 ^{e,B}	10.21 ± 2.35 ^{f,F}	41.63 ± 1.79 ^{g,B}
1.56	25.60 ± 5.06 ^{ef,F}	38.14 ± 0.88 ^{e,CD}	19.34 ± 1.29 ^{e,G}	34.22 ± 1.32 ^{de,DE}	63.78 ± 1.58 ^{de,A}	30.83 ± 1.32 ^{f,E}	39.74 ± 1.29 ^{e,C}	18.36 ± 1.44 ^{e,G}	52.92 ± 0.93 ^{f,B}
3.12	31.82 ± 4.58 ^{de,EF}	38.52 ± 1.05 ^{e,DE}	29.26 ± 3.16 ^{d,FG}	41.85 ± 3.31 ^{d,D}	70.70 ± 1.96 ^{d,A}	34.84 ± 3.31 ^{f,EF}	50.10 ± 5.81 ^{d,C}	24.17 ± 3.10 ^{e,G}	57.59 ± 2.39 ^{ef,B}
6.25	41.22 ± 3.44 ^{d,D}	38.01 ± 2.17 ^{e,D}	32.93 ± 2.88 ^{d,E}	55.79 ± 1.64 ^{c,C}	81.76 ± 0.64 ^{c,A}	43.44 ± 1.61 ^{e,D}	55.04 ± 0.96 ^{d,C}	39.22 ± 3.22 ^{d,D}	66.14 ± 3.56 ^{de,B}
12.5	55.75 ± 0.82 ^{c,D}	46.82 ± 4.5 ^{d,E}	39.86 ± 0.93 ^{cd,F}	62.61 ± 1.53 ^{c,C}	85.38 ± 2.39 ^{bc,A}	58.55 ± 0.15 ^{d,CD}	75.59 ± 2.61 ^{c,B}	42.71 ± 1.07 ^{d,EF}	73.89 ± 3.90 ^{cd,B}
25	71.39 ± 0.75 ^{b,C}	55.19 ± 1.36 ^{c,D}	48.59 ± 2.61 ^{bc,E}	75.66 ± 0.15 ^{b,C}	88.06 ± 0.93 ^{bc,A}	72.24 ± 0.90 ^{c,C}	88.62 ± 3.16 ^{b,A}	53.81 ± 1.04 ^{c,D}	80.25 ± 3.54 ^{bc,B}
50	79.46 ± 1.62 ^{ab,DE}	74.97 ± 0.96 ^{b,EF}	50.01 ± 4.89 ^{b,G}	81.22 ± 3.01 ^{b,CD}	90.78 ± 1.79 ^{ab,A}	79.91 ± .3.01 ^{b,CDE}	87.50 ± 0.83 ^{b,AB}	72.27 ± 1.17 ^{b,F}	84.92 ± 2.54 ^{b,BC}
100	87.19 ± 3.59 ^{a,BC}	85.02 ± 2.15 ^{a,BC}	85.43 ± 5.81 ^{a,BC}	88.93 ± 4.84 ^{a,B}	95.83 ± 4.55 ^{a,A}	87.30 ± 1.28 ^{a,BC}	97.00 ± 2.61 ^{a,A}	80.82 ± 1.56 ^{a,C}	96.46 ± 2.65 ^{a,A}

Results are expressed as mean ± standard errors of triplicates. Means in the same column followed by different lowercase superscript letters are significantly different (p <0.05). Means in the same row followed by different uppercase superscript letters are significantly different (p <0.05).

Table 2: Anti-adhesion activity (% inhibition) of various concentrations of the BS on the adhesion of selective microorganisms in 96-well microtiter plate

Glycoprotein (mg/mL)	*B. subtilis*	*E. coli*	*S. aureus*	*S. marcescens*	*E. aerogenes*	*K. pneumoniae*	*S. faecalis*	*P. vulgaris*	*P. aeruginosa*
0.39	8.19 ± 0.39 ^{g,E}	53.87 ± 3.61 ^{f,A}	15.66 ± 0.71 ^{f,D}	38.73 ± 1.67 ^{f,B}	27.02 ± 1.47 ^{g,C}	7.96 ± 1.42 ^{g,E}	38.56 ± 5.03 ^{h,B}	43.36 ± 2.70 ^{g,B}	31.01 ± 5.75 ^{g,C}
0.781	11.88 ± 0.18 ^{fg,G}	59.20 ± 0.48 ^{e,A}	29.99 ± 2.74 ^{e,E}	42.95 ± 0.94 ^{f,C}	36.10 ± 1.94 ^{f,D}	18.04 ± 0.41 ^{f,F}	49.08 ± 0.88 ^{g,B}	51.81 ± 5.32 ^{f,B}	41.77 ± 1.45 ^{f,C}
1.56	15.07 ± 1.52 ^{f,F}	66.13 ± 3.45 ^{d,A}	50.32 ± 3.69 ^{d,C}	51.31 ± 1.67 ^{e,BC}	40.42 ± 2.48 ^{f,D}	22.45 ± 4.65 ^{ef,E}	55.90 ± 0.69 ^{f,B}	56.67 ± 0.68 ^{e,B}	51.39 ± 1.49 ^{e,BC}
3.12	29.28 ± 1.33 ^{e,E}	70.50 ± 1.99 ^{d,A}	54.35 ± 2.71 ^{cd,C}	53.19 ± 0.86 ^{e,C}	46.76 ± 2.36 ^{e,D}	28.62 ± 3.47 ^{de,E}	59.47 ± 1.82 ^{ef,B}	63.48 ± 0.79 ^{d,B}	54.79 ± 3.90 ^{e,C}
6.25	32.92 ± 0.65 ^{de,F}	78.48 ± 4.57 ^{c,A}	58.40 ± 1.34 ^{c,D}	59.42 ± 0.95 ^{d,D}	52.27 ± 3.29 ^{d,E}	33.29 ± 1.59 ^{d,F}	62.77 ± 1.73 ^{e,CD}	66.96 ± 2.26 ^{d,BC}	70.13 ± 1.24 ^{d,B}
12.5	36.04 ± 0.36 ^{cd,D}	84.58 ± 1.09 ^{b,A}	58.97 ± 0.55 ^{c,E}	62.17 ± 2.30 ^{d,C}	60.58 ± 3.55 ^{c,C}	34.93 ± 2.46 ^{d,D}	70.58 ± 3.43 ^{d,B}	73.14 ± 0.82 ^{c,B}	79.94 ± 4.29 ^{c,A}
25	39.18 ± 3.83 ^{c,E}	89.28 ± 1.83 ^{b,A}	70.91 ± 3.23 ^{b,CD}	70.31 ± 3.02 ^{c,D}	77.65 ± 2.69 ^{b,BC}	42.01 ± 4.34 ^{c,E}	77.07 ± 2.43 ^{c,BCD}	76.01 ± 1.97 ^{c,CD}	83.20 ± 5.08 ^{bc,AB}
50	43.97 ± 1.26 ^{b,F}	94.31 ± 0.90 ^{a,A}	76.25 ± 3.70 ^{b,D}	76.91 ± 3.18 ^{b,D}	83.78 ± 2.41 ^{a,C}	66.82 ± 3.83 ^{b,E}	90.45 ± 6.10 ^{b,AB}	83.33 ± 1.28 ^{b,C}	87.44 ± 0.87 ^{b,BC}
100	50.50 ± 4 ^{a,C}	97.13 ± 1.07 ^{a,A}	88.30 ± 6.57 ^{a,AB}	89.27 ± 6.60 ^{a,BC}	88.37 ± 1.68 ^{a,AB}	88.58 ± 3.93 ^{a,AB}	97.94 ± 1.89 ^{a,A}	92.41 ± 2.67 ^{a,A}	96.92 ± 1.97 ^{a,A}

Results are expressed as mean ± standard errors of triplicates. Means in the same column followed by different lowercase superscript letters are significantly different (p <0.05). Means in the same row followed by different uppercase superscript letters are significant.

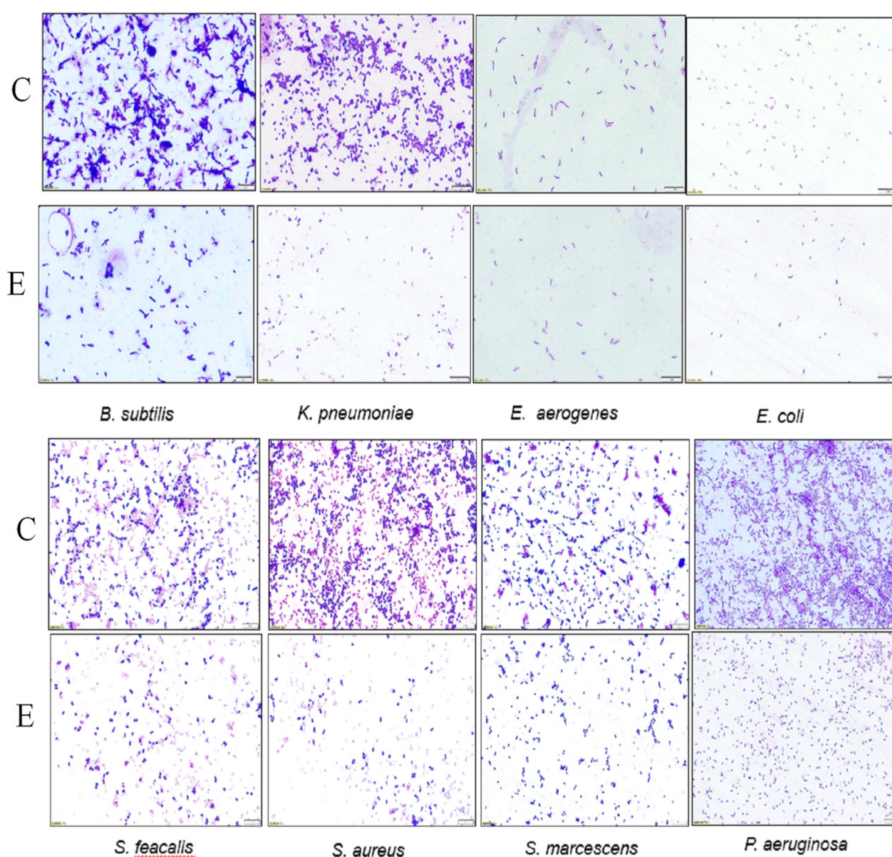

Fig. 3: Antiadhesive activity of BS on microorganisms on the glass slide. 'C': Control without BS coating, and 'E': Experimental slides coated with BS.

microorganisms, depending on the BS concentration and the specific microorganism. As BS concentration increased, both antimicrobial and antiadhesive activities enhanced.

BS modifies the physicochemical properties of surfaces, such as hydrophobicity, reducing adhesion, and biofilm formation on biomaterials. Thus, BS coating can delay biofilm formation initiation.[31] This experiment observed this inhibition of microbial adhesion. Microscopic observation of slides indicated that the BS efficiently disrupted biofilms formed on glass surfaces. Similar results were obtained in another study, where using SNAU01 lipopeptide at 250 µg/mL inhibited biofilm formation by *P. aeruginosa* MTCC 2453 and *E. coli* MTCC 2939 on glass surfaces.[43] The glycoprotein BS (5 mg/mL) from *Lactobacillus agilis* CCUG31450 inhibited the growth of *S. aureus* (20%), *P. aeruginosa* (13.5%), and *S. agalactiae* (11%).[44] However, it did not show antimicrobial activity against *E. coli* and *C. albicans* under the same conditions.

Microbial adhesion to surfaces poses a significant challenge for hospitals. BS's anti-adhesive properties against biofilm producers indicate its potential application as an anti-adhesive agent on medical devices such as catheters and prostheses to prevent microbial infections.[45] BS plays an essential role in preventing biofilm formation on surfaces such as silicon,[46,47] titanium,[48] and polystyrene plates.[49] The risk of infection for patients undergoing urinary catheterization increases by approximately 10% each day. In this investigation, a commercially available medical-grade catheter was used to examine the impact of BS on bacterial biofilms. The results showed that, the BS coating effectively reduces the *P. aeruginosa* biofilm formation. A literature study showed that pre-coating silicone urethral catheters with glycolipid can prevent the growth of *P. vulgaris* NCIM 2027.[33,34] Partially purified extracellular BS from *Leuconostoc mesenteroides*-coated catheters exhibited maximum biofilm inhibition of 54% against *E. coli* and 43% against *P. aeruginosa*.[50] BS produced from *Lactobacillus acidophilus* decreased biofilm amounts by *E. faecalis* and *S. epidermidis* on catheters by 62.2 and 47.3%, respectively, and on microtiter plate wells by 48.2 and 44.6%, respectively.[50]

In vitro cytotoxicity is a critical parameter for biosafety assessment, evaluating the impact of BS on cells before clinical applications.[51,52] The lack of cytotoxic effects is crucial for

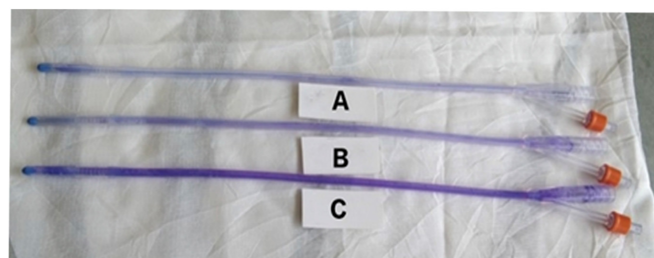

Fig. 4: Antibiofilm activity of BS on medical grade catheter: **(a)** Test showing the anti-biofilm properties of BS against the bacterial strain. **(b)** Positive control shows the anti-biofilm property of SDS against *P. aeruginosa*, and **(c)** Control dark purple indicates the presence of *P. aeruginosa* biofilm

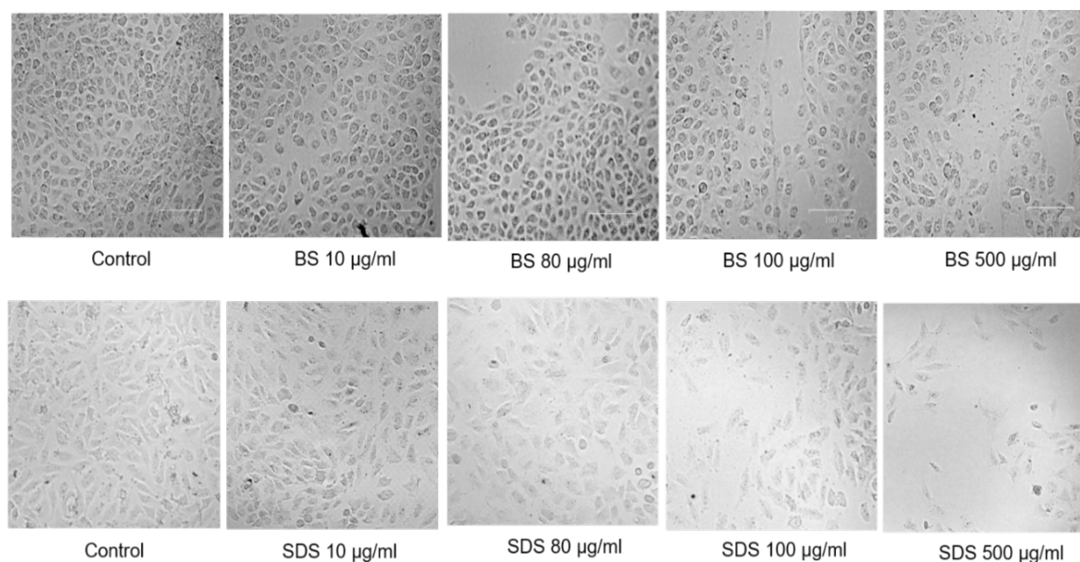

Fig. 5: Morphological observations of Vero cells treated with (a) biosurfactant and (b) SDS under an inverted phase-contrast microscope.

considering applications related to human health and antimicrobial, anti-adhesive, and antibiofilm properties.[53] BS from *Lactobacillus* spp. has shown low cytotoxic effects on human lung epithelial cell lines comparable to commercially available rhamnolipids.[54] The FDA-approved daptomycin, a lipopeptide BS, demonstrates its safety and efficacy for medical applications.[55] Other BSs, such as lactonic sophorolipid and surfactin, also showed low toxicity, making them safe for biomedical applications.[56,57]

CONCLUSION

In conclusion, this investigation illustrates the potent antimicrobial, anti-adhesive, and anti-biofilm properties of BS produced by *D. fermentans* JDP, underscoring its possible utility in the biomedical and industrial domains. *D. fermentans* JDP produced 5.98 gm% of BS under optimized conditions. The presence of BS was confirmed by oil spreading assay, drop collapsing assay and emulsification assay. The BS displayed significant antimicrobial efficacy against Gram positive and Gram negative bacteria, exhibiting a remarkable zone of inhibition for *E. aerogenes*, *K. pneumoniae*, *B. subtilis*, *S. marcescens*, *S. faecalis*, and *S. aureus*. *In vitro* assessments for biofilm inhibition indicated that the BS effectively diminished biofilm formation, especially with increased concentrations. The BS accomplished a 97% inhibition against *S. faecalis*, suggesting its robust antiadhesive properties. Comparable inhibitory impacts were also observed for *P. aeruginosa*, *E. aerogenes*, and *S. marcescens*. Furthermore, the antibiofilm efficacy of the BS was validated on glass slides and medical-grade catheters, where it remarkably disrupted biofilm formation by *P. aeruginosa*. The cytotoxicity evaluation on Vero cells revealed that the BS exhibited biocompatibility at 200 µg/mL concentrations, with an IC_{50} value of 217.5 µg/mL, indicating its safety for prospective biomedical uses. The results propose that the BS derived from JDP9 could serve as a valuable component in formulating antimicrobial coatings for medical apparatus, thereby diminishing the likelihood of biofilm-related infections.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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